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14. ABSTRACT During this project period, we employed in vivo and in vitro approaches to determine whether the endogenous cannabinoid system can be targeted to treat breast cancer. The first objective of this work was to develop a preclinical model to induce mammary tumors in mice and determine whether elevating levels of the endogenous cannabinoid anandamide through the use of mice lacking fatty acid amide hydrolase (FAAH), the primary catabolic enzyme responsible for degrading this endogenous cannabinoid, would show decreased tumorigenesis. The carcinogen, DMBA, produced the appearance of tumors in the mammary region of female control C57Bl/6 mice and FAAH knockout mice. However, the tumors could not be conclusively identified as being breast cancer. The second objective of this project was to evaluate interactions of cannabinoids with radiation in MCF-7, MDA-MB-231, and 4T1 breast tumor cell lines. Interestingly, the high efficacy synthetic cannabinoid agonist WIN55,212-2, but not its stereoisomer WIN55,212-3 or the phytocannabinoids Δ9-tetrahydrocannabinol (THC) or cannabidiol (CBD), significantly enhanced the effects of radiation in decreasing cell viability. The enhanced effects of combination of WIN55,212-2 and radiation on breast cancer suggest the possibility that this type of strategy may hold promise to treat this disease.					
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INTRODUCTION:

The endogenous cannabinoid (eCB) system consists of two G-protein coupled receptors (CB₁ and CB₂), two membrane-derived lipids anandamide (AEA) and 2-arachidonylglycerol (2-AG), and enzymes regulating the biosynthesis and hydrolysis of these eCBs¹. Presently, two FDA -approved cannabinoids, marinol (oral Δ^9 -tetrahydrocannabinol (THC))^{2,3} and Cesamet (oral Nabilone)^{4,5} are prescribed to treat emesis and nausea associated with cancer chemotherapeutic agents. Cannabinoid receptor agonists have been shown to alter tumor growth and progression in *in vitro* and preclinical cancer models⁶⁻¹⁰; however, little work has looked at the involvement of the eCB system in tumor development. The primary objective of this project was to test whether increasing levels of endogenous anandamide through deletion of its primary catabolic enzyme fatty acid amide hydrolase (FAAH) would prevent tumor genesis as well as produce antiproliferative^{11,12} and decrease tumor invasiveness caused by the carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA). Because, cancer patients can be treated with oral cannabinoids along with chemotherapy or radiation, a second objective of this research was to determine whether cannabinoid receptor agonists or antagonists in combination with chemotherapy or radiation would reduce breast cell proliferation in cell culture. A cell culture system was employed because of significant challenges associated with the DMBA-induced mouse breast cancer model. Specifically, the protracted period of time required to obtain palpable tumors (> 40 weeks) and the difficulty in determining the tissue of origin due to small the separation between the skin, mammary tissue, and abdominal muscle tissue required that we utilize an *in vitro* approach. The report herein describes the results of our studies examining the impact of: 1) DMBA-induced mammary tumorigenesis in FAAH (-/-) mice vs. wild type mice; and 2) the synthetic cannabinoid receptor agonist WIN55,212-2 in combination with radiation or adriamycin on breast cell proliferation *in vitro*.

BODY

Study 1. Impact of FAAH deletion in the murine DMBA-induced mammary tumor model (model development is included in 30-09-2009 annual report)

Methods

Subjects. Subjects consisted of female mice on a C57BL6/J background that included the following genotypes: FAAH (+/-) mice: equivalent to wild type mice, FAAH (-/-) mice: genetic deletion of FAAH (10 fold elevated AEA levels), and FAAH-neural specific (NS) mice: genetic deletion of FAAH in non-neuronal tissue (i.e., elevated AEA levels)

Procedure. The subjects were subcutaneously implanted with 25 mg progesterone pellets and three weeks later were dosed with dimethylbenzanthracene (DMBA; 50 mg/kg) administered via gavage once a week for 4 weeks. The DMBA was dissolved in cottonseed oil and administered via gavage. The mice were observed twice/week for the development of tumors. Once the tumor load reached 1 cm³ the animal was humanely euthanized by CO₂ affixation followed by cervical dislocation. The tumor tissue was excised and fixed in a 10% formalin solution. The tissue sections were tested immunohistologically using: Hematoxylin and Eosin stain, p63, Cytokeratin 5/6, Cytokeratin 7, Mucicarmine, and PAS diastase.

Results

As shown in Figure 1, administration of DMBA and progesterone caused tumorigenesis in C57 mice. However, there was no significant effect of FAAH genotype in the incidence of tumor growth. Based on the histology, the tumors fell into the following four categories (Figure 2):

1. Adenocarcinoma (mammary)
2. Squamous cell carcinoma (skin)
3. Myosarcoma (muscle)
4. Uncharacterized (more than one phenotype in same tissue sample)

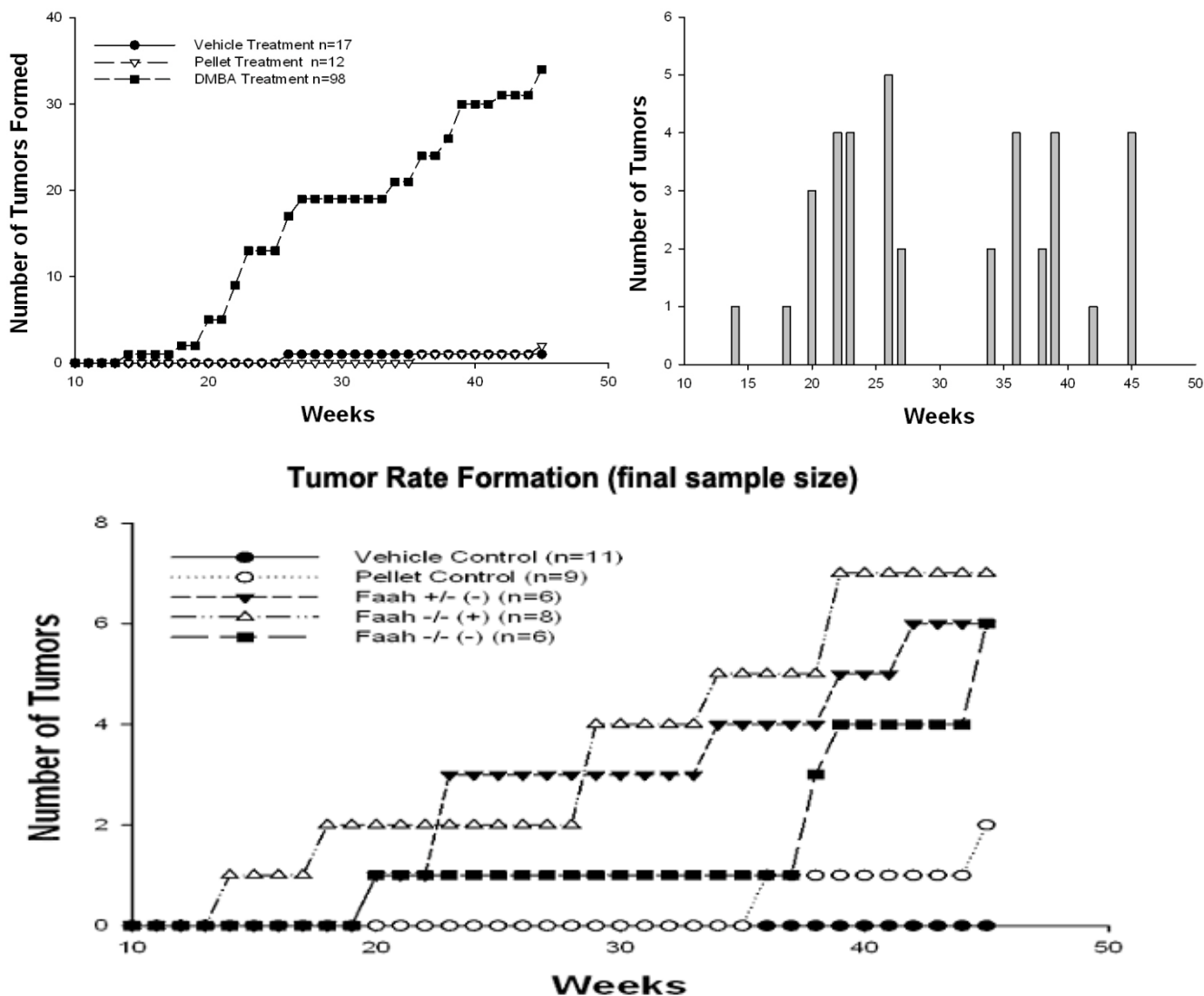
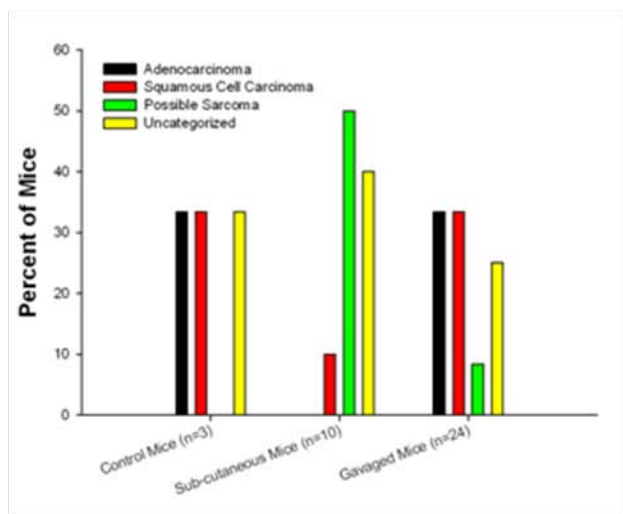
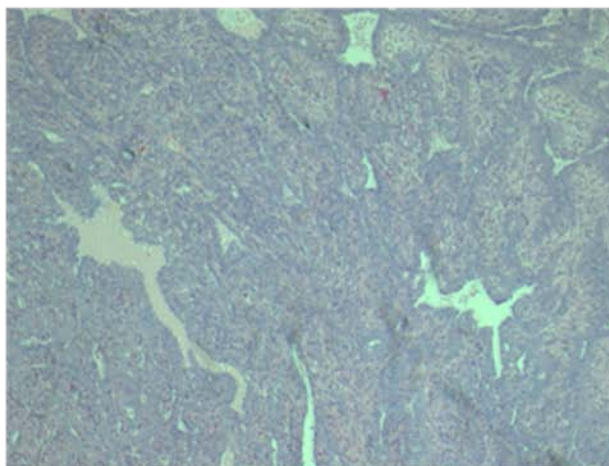


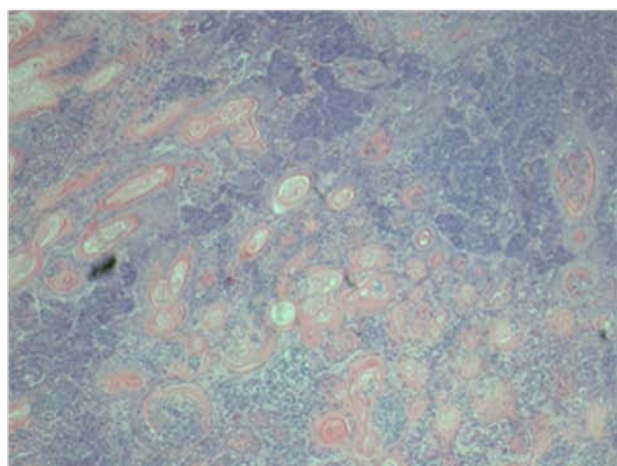
Figure 1. Vehicle and pellet treatments showed no or low numbers of tumor production. All DMBA-treated mice showed consistent and steady tumor formation over the 45 week observation period. No individual week showed a higher level of tumor formation than any other. No significant differences in rate of tumor formation were observed across any of the genotypes tested.



Adenocarcinoma



Squamous Cell Carcinoma



Myosarcoma

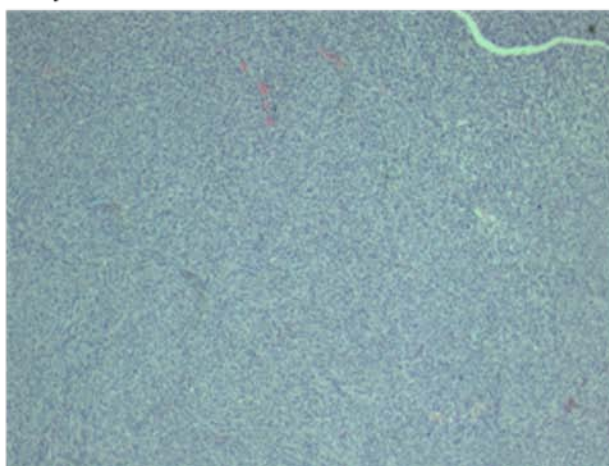


Figure 2. Histology analysis pooled the tumors into 4 categories: 1) Adenocarcinoma; 2) Squamous Cell Carcinoma; 3) Myosarcoma; 4) Unknown (multiple phenotypes in same tissue sample)

Because DMBA-induced tumors required months to develop, attempts were made to derive cell lines from the excised tumors in order to test whether different cannabinoids would produce antiproliferative effects. The following three cannabinoids were evaluated: THC (the primary active psychoactive constituent present in marijuana), cannabidiol (CBD: a marijuana-derived cannabinoid that lacks psychomimetic effects, and WIN55,212-2 (Win2; a high efficacy synthetic cannabinoid receptor agonist). Four of the 19 tumors we excised for culture, grew without contamination. The experiment was conducted on BR15 cells (see Figure 3) that were derived from a wild type control mouse (subject #BR15). For comparison the antiproliferative effects of the test compounds were also evaluated in MCF7 cells. As shown in Figure 4, each of the treatments reduced

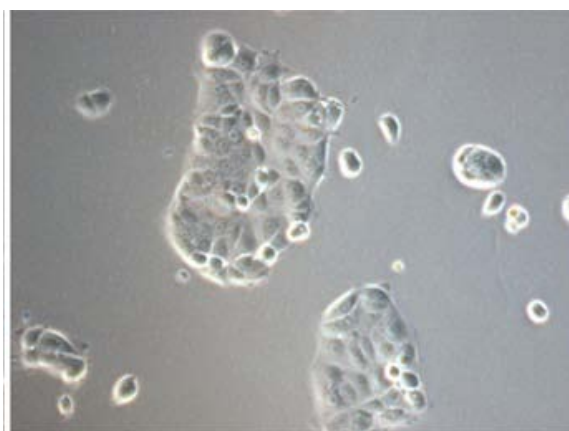


Figure 3. Photomicrograph of tumor cells derived from a DMBA-treated wild type mouse (BR15).

proliferation in both the DMBA-derived tumors and MCF7 cells with the following relative potency: Win2 > CBD > THC.

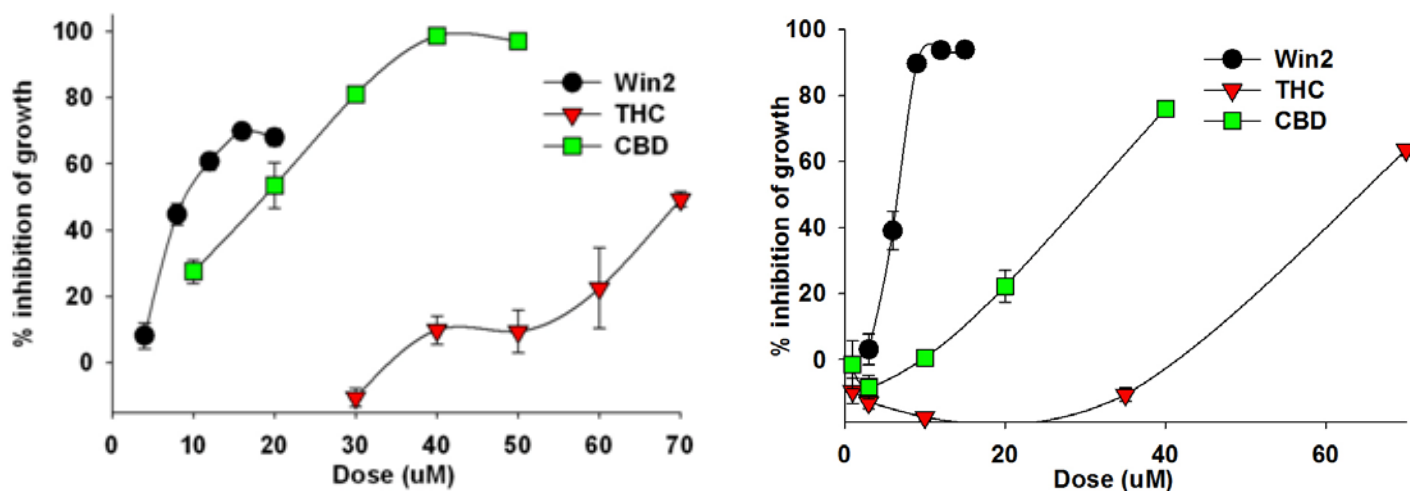


Figure 4. Antiproliferative effects of Win2, THC, and CBD in BR15 cells, derived from tumors excised from DMBA-treated mice. BR15 cells displayed similar sensitivity to these cannabinoids as MCF7 cells, an established breast cancer cell line.

Because BR2 cells were derived from C57BL/6J inbred mice, this cell line represents a unique opportunity to investigate tumor proliferation in a mouse strain that serves as the background of many knockout and knock-in mouse lines, to reduce the likelihood of immune rejection. Nonetheless, the inability to ascertain unequivocally that these cells were breast-derived precludes their use as a model of breast cancer.

Study 2. Interaction between cannabinoids and radiation in breast tumor cells

An area that has received surprising little attention relates to the utilization of cannabinoids in combination with conventional chemotherapeutic drugs or radiation. This issue would appear to be of considerable importance both for the application of the cannabinoids to ameliorate side effects of these treatments as well as their possible incorporation into current clinical protocols that generally involve both chemotherapy and radiation treatment. In this context, a report suggested that the immune-suppressive function of the cannabinoids would likely limit their antitumor activity and by extension, the activity of conventional drugs or radiation used for cancer treatment. However, no study of which we are aware has evaluated the combination of cannabinoids with drugs or radiation against tumor cells either in cell culture or an animal model. Consequently, the current studies were designed to address the question of whether cannabinoids alter the effects of ionization radiation three distinct breast cancer cell lines.

Methods

Cell lines. MCF-7, MDA-MB-231, and MCF-10a cells were obtained from ATCC. 4T1 cells were obtained from Caliper. MCF-7, MDA-MB-231 and 4T1 cells were cultured in RPMI media with 1% pen/strep solution, 5% fetal bovine serum and 5% bovine calf serum. MCF-10a cells were cultured in DMEM/F12 media supplemented with 1% pen/strep solution, 10% horse serum, insulin 10ug/ml, cholera toxin 100ng/ml, EGF 20ng/ml, and hydrocortisone 500ng/ml.

Drug treatments. All cannabinoid treatments were administered for 24 h before being washed. Radiation treatments were given at the beginning of the 24 h treatment unless otherwise stated. All cannabinoid antagonists were given at the same time as the agonist. Chloroquine and Necrostatin-1 were administered the same time as the cannabinoids and left on for 48 h. Glutathione was administered 24 h before cannabinoid or radiation treatment and left on for 48 h, for a total of 72 h.

Clonogenic Survival. Cells were trypsinized and diluted into a low density solution and 200 cells were plated per well into a 6 well plate and allowed to adhere overnight. Media was changed once weekly and cells were allowed to grow 10-14 days or until control colonies were visually observed.

Determination of viable cell number. Cells were plated into 6 well plates at a density appropriate to the time point and growth rate of the cell line. After treatments were completed, the cells were harvested and counted using a hemocytometer or Invitrogen Countess automated counter. A 1:1 dilution with trypan blue was used to assess cell viability.

Crystal Violet Assay. Cells were plated into 96 well plates at a density appropriate to the time point and growth rate of the cell line. After treatments were completed cells were washed with PBS and fixed with methanol. The cells were then stained with a 0.1 crystal violet solution and washed with PBS to remove background. Crystal violet was solubilized with a 50% ethanol and Na⁺Citrate solution. Samples were read on a microplate reader.

Cell cycle analysis. After treatment, the samples were harvested, supernatant was removed and then fixed in 3.7% formaldehyde. Cells were then maintained in 90% methanol at 4°C for at least 10 min or until all samples had been collected. In order to stain, the methanol was removed and cells were incubated overnight in staining solution: PBS, 0.1% triton-x 100, 50ug/million cells Propidium Iodide, and 100ug DNase free RNase. Samples were measured on a canto BD Biosciences flow cytometer with an emission wavelength of 617nm.

Annexin V and PI. After samples were harvested the supernatant was removed and they are washed in PBS. Cell pellets were then suspended in staining solution: 10x binding buffer diluted to 1x, Annexin V solution diluted to 5%, Propidium Iodide solution diluted to 5%. Staining solution was made from the BD Bioscience Annexin V and PI staining kit. Samples were measured on a canto BD Biosciences flow cytometer with an emission wavelength of 617nm and 520nm.

Results

Studies of receptor mediated antiproliferative action of the cannabinoids in breast tumor cells.

Initial experiments were conducted to evaluate receptor-mediated growth inhibition by Win2, THC, cannabidiol (CBD) and nabilone in p53 wild-type MCF-7 breast tumor cell line at near-maximal growth inhibitory concentrations (CBD at 40μM, Nabilone (NAB) at 50μM, and THC at 80μM). Figure 5 (left panel) shows that the respective CB1 and CB2 receptor antagonists, SR141716 and SR144528, interfered with growth inhibition by the cannabinoids. Similar data were generated with two additional commonly used breast tumor cells lines, the p53 mutant MDA-MB231 human breast tumor cell line that is often considered to be a model of triple negative breast cancer and the murine p53 null 4T1 breast tumor cell line (Figure 5 center and right panels). The capacity of the CB1 and CB2 receptor antagonists to prevent growth inhibition by the various cannabinoids varied across the different cells lines and in no case was growth inhibition completely reversed. Nevertheless, these findings are consistent with the notion that CB1 and CB2 receptors contribute to the inhibitory actions of these compounds on growth.

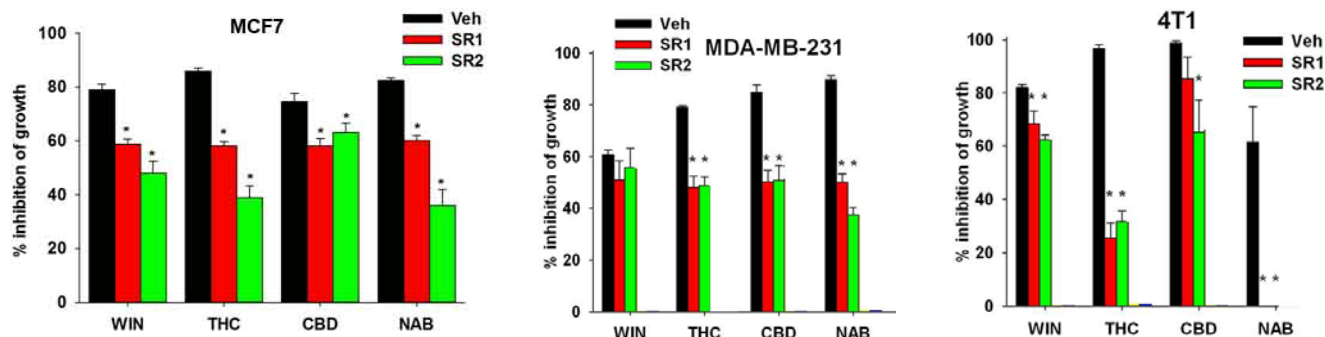


Figure 5. MCF-7, MDA-MB-231, and 4T1 cells were treated with Win55,212-2 (Win; 12uM), Cannabidiol (CBD; 36uM), Nabilone (NAB; 60uM) or THC (84uM) in the Crystal Violet assay. Cells were also treated with vehicle (Veh; media), rimonabant (SR1; 1uM) or SR144528 (SR2; 1uM). *p<0.05 vs. Veh.

As the data presented in Figures 5 might be interpreted to suggest that the antiproliferative activity of Win2 could be occurring through a nonspecific site of action, we performed dose response studies in the three breast tumor cell lines using both Win2 and its inactive enantiomer, Win55,212-3 (Win3). Figure 6 shows that Win2 inhibited growth with far greater potency and efficacy than Win3 in each cell line.

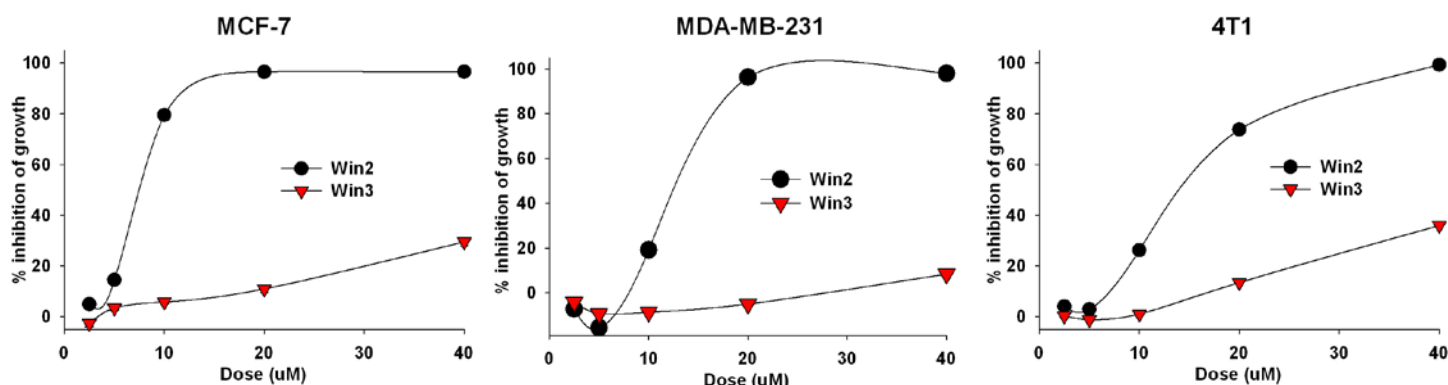


Figure 6. MCF-7, MDA-MB-231, and 4T1 cells were treated with Win55,212-2 (Win2) or its inactive stereoisomer Win55,212-3 (WIN3). Win2 was significantly more potent than Win3 in inhibiting growth of each cell line.

Interaction between the cannabinoids and radiation

Although there is extensive preclinical evidence for the potential of cannabinoids to suppress growth and/or invasion and metastasis in various experimental cancer models, it is evident that translation of these agents to the clinic would not involve their utilization as monotherapy, but that they would be administered in association with conventional antitumor drugs and/or radiation therapy. Moreover, THC and nabilone are used to treat nausea and vomiting associated with these therapies. Consequently, studies were performed to evaluate the influence of the various cannabinoids on the response to radiation.

Strikingly, the combination of Win2 and radiation was more effective than either treatment alone utilizing a clonogenic survival assay (Figure 7, left panel). This finding was confirmed in MDA-MB-231 (Figure 7, center panel) and 4T1 (Figures 7, right panel) breast tumor cell lines.

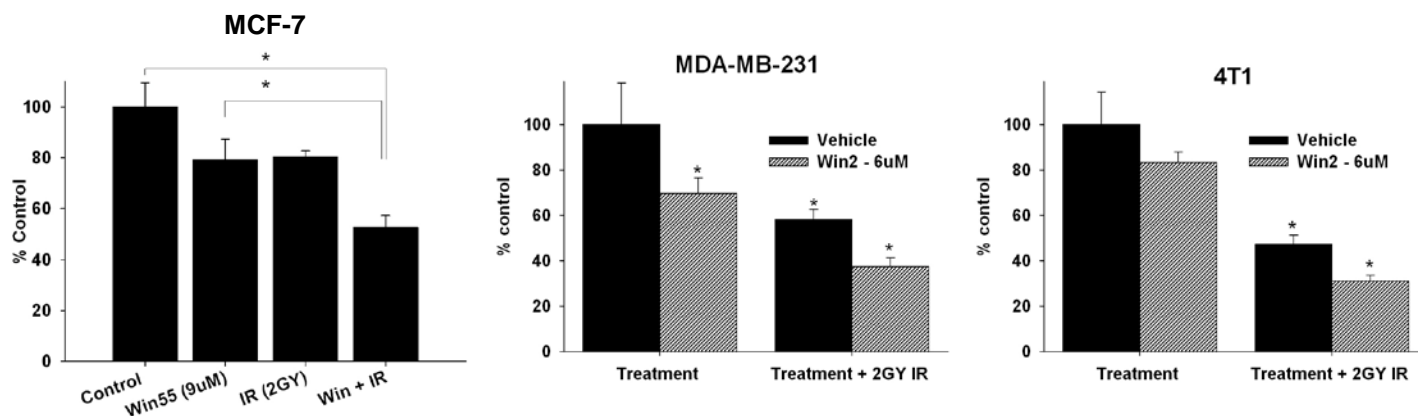


Figure 7. Augmented effects of combined WIN55,212-2 (WIN55 or Win2) and radiation in MCF-7 cells, MDA-MB-231 cells, and 4T1 cells, as assessed in the clonogenic survival assay. Concentration of Win55,212-2 is indicated in the figure with a single treatment of 2Gy radiation alone or in combination. * $p < 0.05$.

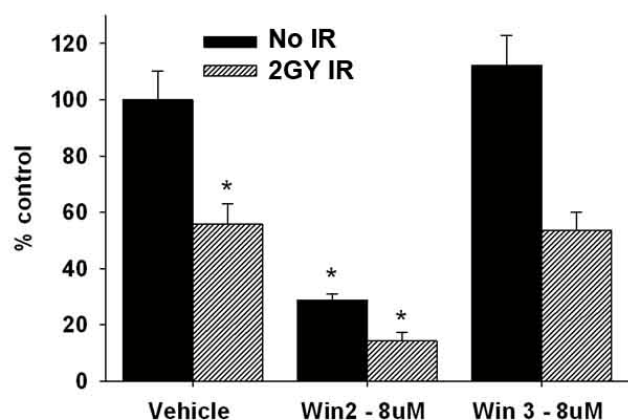


Figure 8. WIN55,212-2, but not its stereoisomer WIN55,212-3 augmented the effects of radiation in MCF-7 cells. * $p < 0.05$ vs. the Vehicle-No IR condition.

performed in the MCF-10A cell line that is considered to be a model of normal breast epithelial cells. Although these cells appeared to be exceptionally sensitive to radiation, the addition of WIN55,212-2 did not enhance radiation sensitivity, which is consistent with a selective action against the breast tumor cells (Figure 9). However, it should be noted that the effects of radiation were really high in the MCF-10a cells, which suggests that the experiment should be repeated using a lower dose of radiation (e.g., 1Gy) to rule out the possibility of a ceiling effect.

To determine whether other cannabinoids would also interact with radiation, cannabidiol (10, 25, and 40uM) or THC (30, 45 and 70uM) were administered to MCF-7 cells in combination with 2Gy radiation (Figure 10).

In order to test whether the augmented effects of this cannabinoid agonist and radiation were nonspecific, we compared the effects of radiation in combination with WIN55,212-2 or its inactive stereoisomer WIN55,212-3. Figure 8 indicates that WIN55,212-3 failed to produce any effects on its own or enhance the effects of radiation. In contrast, the combination of WIN55,212-2 and radiation replicated the effects found in Figure 7 (left panel), which suggests target specificity.

To assess the potential selectivity of this treatment strategy, similar combination experiments were

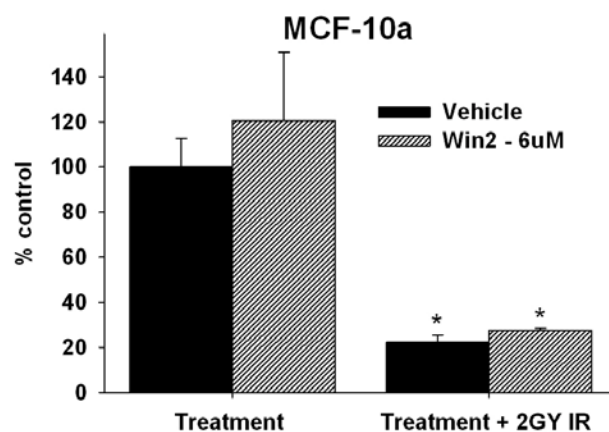


Figure 9. WIN55,212-2 (Win2) did not augment the effects of single dose of 2Gy radiation in MCF-10a cells. * $p < 0.05$ vs. vehicle.

Curiously, the combination treatment did not produce significantly greater effects than either the drug or radiation treatment alone.

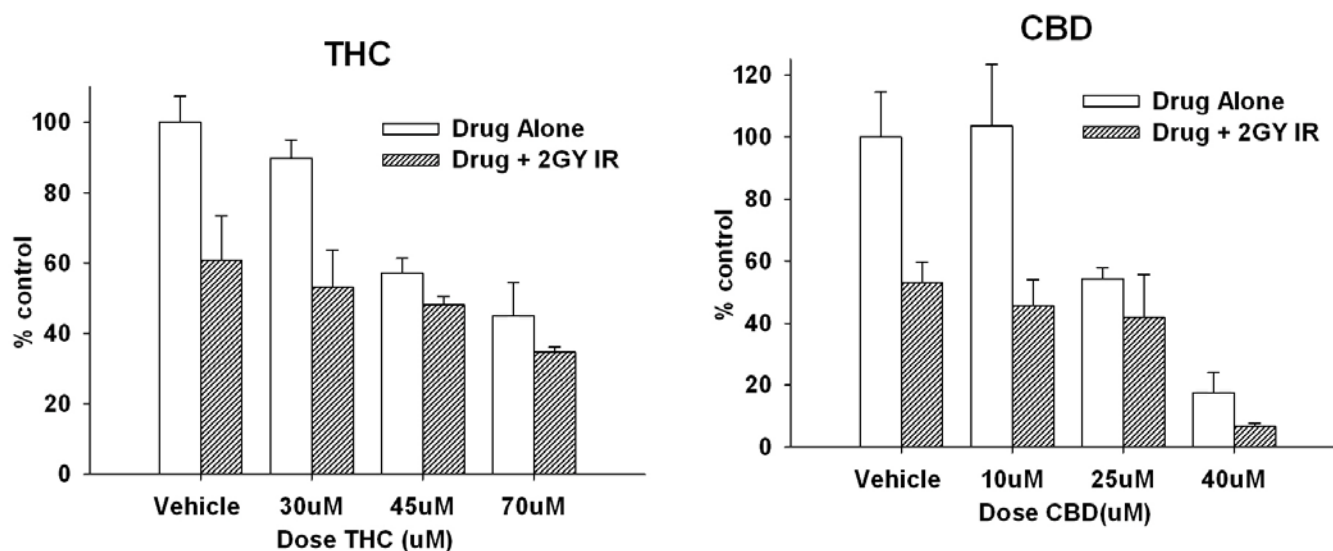


Figure 10. Neither THC (left panel) nor cannabidiol (right panel) enhanced the effects of radiation alone in MCF-7 cells.

Cell cycle analysis was performed using flow cytometry. At 24 h, cells treated with either WIN55,212-2 alone or in combination with radiation accumulated primarily in the G1 phase, with an evident decline in the S phase population (Figure 11, left panel). In contrast, at 48 h, the G1 accumulation disappeared and a significant increase in the subG1 population became evident (Figure 11, right panel).

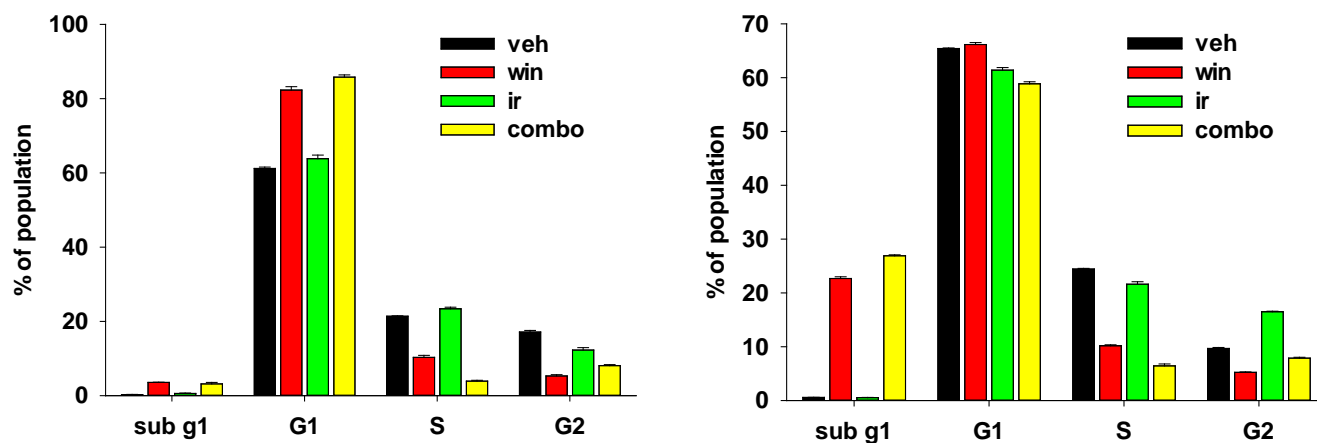


Figure 11. MCF-7 cells treated with 8uM Win55,212-2 (win) or 1 dose of 2Gy radiation either alone or in combination were stained with Prodidium Iodide to determine the amount of DNA content for cell cycle analysis. At 24 h (left panel) and 48 h (right panel) significant changes were found in distribution of cells treated with Win55,212-2 or Win55,212-2+radiation.

This pattern of results generally indicates the occurrence of apoptosis (and/or possibly necrosis). However, Annexin V/ PI staining failed to support the promotion of either apoptosis or necrosis by WIN55,212-2 alone or in combination with IR (Figure 12). On the other hand, staurosporine, used as a positive control, provided evidence of apoptosis.

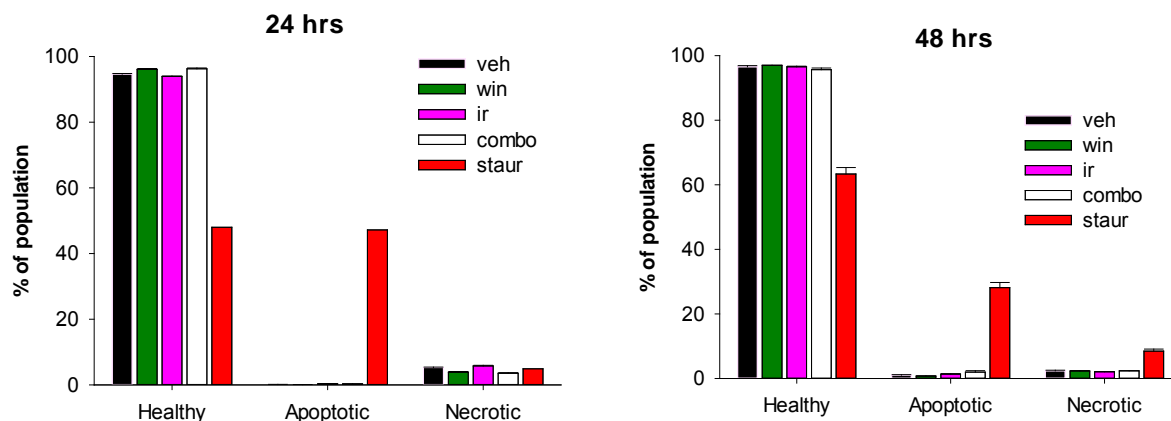


Figure 12. Annexin V and PI staining quantified by flow cytometry at 24 h (left panel) and 48 h (right panel) in cells treated with 8uM Win55,212-2 or 1 dose of 2Gy radiation either alone or in combination. 1uM Staurosporine for 24 h was used as a positive control. No significant changes found between the treatment conditions.

Interestingly, MCF-7 cells treated with WIN55,212-2 alone or in combination with radiation displayed a unique vacuolization (Figure 13) that could be consistent with necrosis.

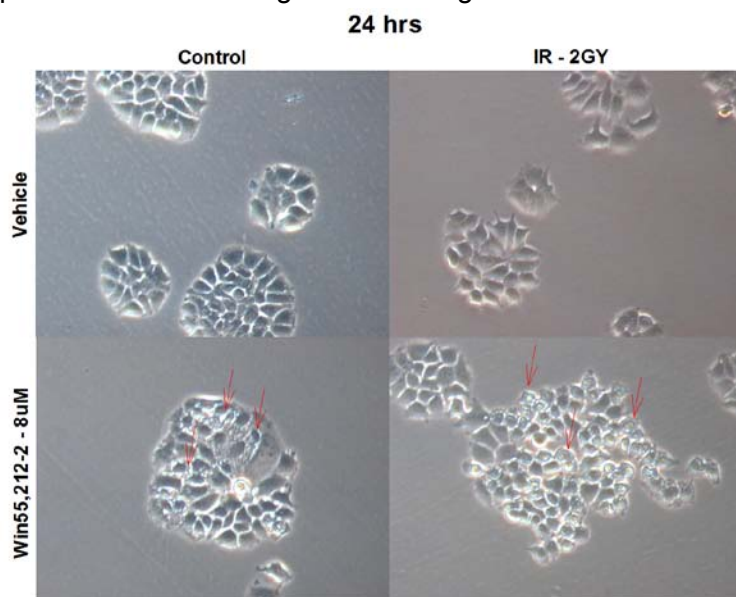


Figure 13. Morphological observations at 24 h in MCF-7 cells treated with 8uM Win55,212-2 or a single dose of 2 Gy radiation either alone or in combination.

KEY RESEARCH ACCOMPLISHMENTS

- Administration of DMBA and progesterone caused tumorigenesis in C57 mice.
- No differences in the incidences of tumors were found between FAAH (-/-) and (+/+) female mice.
- Cancer cells derived from DMBA-treated C57BL/6J mice proved to possess similar sensitivity to the antiproliferative effects to WIN55,212-2, THC and cannabidiol as found in MCF-7 cells
- The antiproliferative effects of WIN55,212-2 in MCF-7, MDA-MB-231, and 4T1 cells were stereoselective and were partially attenuated by CB1 and CB2 receptor antagonists.
- WIN55,212-2, but not its stereoisomer WIN5,212-3, enhanced the effects of radiation in all tumor lines
- Neither THC nor CBD augmented or attenuated the effects of radiation in three breast cancer cell lines.

REPORTABLE OUTCOMES

- 2011 Era of Hope Meeting. Poster Presentation. "Role of the Endogenous Cannabinoid System in a Murine Model of Breast Cancer"
- 2010 International Cannabinoid Research Symposium. Poster Presentation. "Cell line specific enhancement of sensitivity to Adriamycin by phytocannabinoids in breast cancer"
- 2010 Virginia Academy of Science. Student Presentation. "The Full Agonist WIN55,212-2 Exerts Growth Inhibitory Effects Through A Cannabinoid Receptor Independent Mechanism"
- 2010 Sean Emery was awarded a Department of Defense predoctoral training grant DOD BC074355 to continue work on this project towards his PhD degree.

CONCLUSIONS:

Two significant challenges were faced using the DMBA-induced mouse model of tumorigenesis. First, although palpable tumors formed following treatment in the mammary region of female mice, the tissue of origin could not be definitively ascertained due to small the separation among the skin, mammary tissue, and abdominal muscle tissue. Thus, appropriate caution must be applied in interpreting results from this model as a general paradigm of tumorigenesis and not specifically as a model of breast cancer. Second, the prolonged period of time required to obtain palpable tumors (> 40 weeks) renders this model infeasible for high throughput studies. However, tumor cells were derived from C57BL/6J inbred mice and underwent several passages. This cell line, called BR15, represents a unique opportunity to investigate syngeneic tumor proliferation in the C57BL/6J mouse strain that serves as the background of many knockout and knock-in mouse lines; thus reducing the likelihood of immune rejection. Indeed, the antiproliferative potency of three cannabinoids was similar between BR15 tumor cells and MCF-7 breast cancer cells.

The most significant finding of this research is that combination of the synthetic cannabinoid WIN55,212-2 and ionizing radiation significantly enhanced the response antiproliferative effects of three breast tumors lines. The finding that this compound's stereoisomer did not augment the effects of radiation suggests that WIN55,212-2 is acting at a specific target or targets. Interestingly, THC, which is prescribed in oral form to prevent nausea and vomiting in patients being treated for breast, did not alter the effects of ionizing radiation. This lack of effect is relevant because it suggests that THC may not interfere with radiation therapy. Although the DOD support has terminated for this project, studies are continuing to identify the mechanistic basis for the interaction of select cannabinoids with radiation in breast cancer.

Personnel receiving financial support during the course of this grant.

Aron H. Lichtman
Sean Emery
Kelly Long
Qing Tao
Irina Beletskaya

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